Overexpression of the HOX4A (HOXD3) Homeobox Gene in Human Erythroleukemia HEL Cells Results in Altered Adhesive Properties

By Yasushi Taniguchi, Noriyuki Komatsu, and Tetsuya Moriuchi

We previously isolated the human homeobox gene HOX4A (HOXD3) on chromosome 2 from a human genomic library and determined its nucleotide sequence. In the present study, expression of the HOX4A gene was investigated in human hematopoietic cell lines. Reverse transcriptase-mediated polymerase chain reaction analysis showed that the HOX4A gene was expressed in erythroleukemia HEL and K562 cells but not in promyelocytic leukemia HL-60 cells. To study the role of the HOX4A gene in erythropoiesis, expression vectors containing the HOX4A gene in the sense or antisense orientation were introduced into HEL cells. The sense transfectants overexpressing the HOX4A gene formed aggregates, which were composed of densely associated cells adhering to tissue-culture dishes, whereas the parental HEL cells and antisense transfectants adhered poorly to the dishes. Furthermore, the sense transfectants overexpressing the HOX4A gene attached more efficiently to fibronectin and collagen than did the antisense transfectants and parental HEL cells. Northern blot analysis showed that integrin β3 mRNA levels were significantly increased in the HEL cells overexpressing the HOX4A gene, whereas the integrin αβ and αIβb mRNA levels did not show a distinct correlation with HOX4A mRNA levels. Fluorescence-activated cell sorting analysis showed that the sense transfectants overexpressing the HOX4A gene expressed increased levels of integrin αIβb (GP IIB-IIIa) complex as compared with the parental HEL cells and antisense transfectants. These results implicate the homeobox gene HOX4A in the regulation of cell adhesion processes.

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RNA preparation and reverse transcriptase-mediated polymerase chain reaction (RT-PCR). To isolate cytoplasmic RNA, cells grown in tissue culture were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 140 mmol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L dithiothreitol, and 1,000 U/ml placental RNase inhibitor (Toyobo, Osaka, Japan). Cytoplasmic RNA samples were prepared according to the method described in the textbook with a slight modification. RT-PCR was performed with an RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT). A total of 1 μg of cytoplasmic RNA was reverse-transcribed using random hexanucleotides as primers. The obtained cDNA was amplified in a 98-μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl₂, 10 mmol/L primer F (forward) and R (reverse), and 2 U Taq polymerase. PCRs were run in the Iwaki thermal sequencer TSR-300 (Iwaki Glass) at 95°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute for 20, 25, 30, 35, or 40 cycles for the HOX4A cDNA, and for 25 cycles for the cytoplasmic β-actin, integrin β1, and integrin β3 cDNAs, followed by 72°C for 10 minutes. The PCR products were separated by 2% agarose or 7.5% polyacrylamide gel electrophoresis (PAGE). Primer pairs used for PCR amplification were as follows: HOX4A, primer F (5'-CTTCCTGCACTGGAACACCACTCC-3', nucleotides 1683-1705) and primer R (5'-ACTCGCTTCACATTCCGACCCGG-3', nucleotides 3940-3962)24; cys-actin, primer F (5'-GGGAGAATCTGTCGGCACAACACCTTCAC-3', nucleotides 377-401) and primer R (5'-TCTCCTTAAATGTCAACGAGATCCT-3', complementary to nucleotides 1191-1215)25; integrin β1, primer F (5'-CATTACCAAGGTAAGAATGGCGGAG-3', nucleotides 2110-2134) and primer R (5'-CTCCTACTTGGATGTCCTCCAG-3', complementary to nucleotides 2486-2492)26; integrin β3, primer F (5'-AGCCTTGAAGGACACTGGCAAGATGC-3', nucleotides 1979-2003) and primer R (5'-GAGCGCTGACGATCCTTCCAGG-3', complementary to nucleotides 2315-2339)27; and integrin αIIb, primer F (5'-GAGGCTCCTTAGATCCTCCAGG-3', nucleotides 2704-2728) and primer R (5'-CTTGAAGAAGGCGGACCTTTCCAGT-3', complementary to nucleotides 3052-3076).28 To prevent genomic DNA contamination, primers F and R were designed to frame a sequence that crosses an intron on the gene. Primers were synthesized on an Applied Biosystems model 391 DNA synthesizer (Applied Biosystems, Foster City, CA).

Cloning and sequencing. RT-PCR products were cloned directly into the pT7blue T-vector DNA by using the pT7blue T-vector kit (Novagen, Madison, WI). The nucleotide sequence of the fragments was determined by the dideoxy-chain termination method29 using synthetic primers.

Preparation of DNA probes. The probe that covers the nucleotide region encoding an N-terminal part of the HOX4A protein was prepared from the cloned HOX4A gene.30 A 248-bp EcoRI fragment in the pBR322 plasmid was used as a probe. The fragment was purified from agarose gels and was labeled with [γ-32P]-deoxyctydine triphosphate (3,000 μCi/mmole) using an Amersham random primer DNA labeling kit (Amersham, Arlington Heights, IL).

Southern and Northern blot hybridization. Hybridization was performed as described previously.29 RT-PCR products and genomic DNA were separated by 2% and 0.7% agarose gel electrophoresis, respectively, and were transferred onto nylon filters. The filters were hybridized at 42°C for 16 hours with labeled DNA probes, then washed at 50°C in 1× SSPE (180 mmol/L NaCl, 10 mmol/L NaH₂PO₄, and 1 mmol/L EDTA) containing 0.1% sodium dodecyl sulfate. A total of 20 μg of cytoplasmic RNA was electrophoresed on 1% agarose-formaldehyde gels, and the bands were transferred onto nylon filters that were then hybridized at 42°C for 16 hours with labeled DNA probes. The filters were washed at 50°C in 1× SSC (150 mmol/L NaCl and 15 mmol/L sodium citrate) containing 0.1% sodium dodecyl sulfate. The DNA and RNA blots were subjected to autoradiography with Kodak XRP-5 film (Eastman Kodak, Rochester, NY) at −80°C. The radioactive band intensities on the filters were measured by Bio-Image Analyser BA100 (Fuji Film, Kanagawa, Japan).

Construction of plasmid vectors for transfections. The HOX4A genomic DNA, including the full coding region and intron, was inserted into a mammalian expression vector, pMAMneo (Clontech, Palo Alto, CA), in the sense orientation, according to the following processes. A 248-bp Sph I fragment (nucleotides 1385-1632) was isolated from the HOX4A genomic clone, inserted into Sma I-Sph I sites of pUC119, and excised as a 262-bp EcoRI-Sph I fragment (fragment α). A 3101-bp Sph I-BamHI fragment (nucleotides 1633-4733, fragment β) was obtained from the HOX4A genomic clone. EcoRI-BamHI sites in the multiple cloning sites of Brescript II (Stratagene, La Jolla, CA) were changed to BamHI-EcoRI sites. (4) Fragments α and β were ligated and cloned into BamHI-EcoRI sites of Brescript II. (5) A 3418-bp insert was excised as a Xba I-Xho I fragment and inserted into Nhe I-Xho I sites of pMAMneo in the sense orientation [pMAMneo-HOX4A(+); an antisense construct was made by skipping the third step [pMAMneo-HOX4A(-)].

Transfections and cell cloning. Transfections were performed using cloned cells from a single colony and a Lipofectin kit (Boehringer, Mannheim, Germany). A total of 200 μL of a mixture consisting of 170 μL HEPES buffer solution containing 20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 5 or 10 μg of plasmid DNA, and 30 μL of transfection reagent were added to 5 mL of culture medium in which cells were suspended at a concentration of 10⁶ cells/mL. The cells were maintained in a CO₂ incubator at 37°C. The following day, the cells were washed using fresh culture medium and cultured in a medium containing 600 μg/mL G418 (GIBCO, Grand Island, NY). After culturing the cells for 2 weeks in G418, surviving cells were isolated, and 50 cells were seeded in dishes (10 cm in diameter). The cells were cultured for 6 days, and small independent colonies, originating from single cells, were isolated with a micropipet. In all experiments using the transfectants, dexamethasone was added to the culture medium at a concentration of 2 μmol/L.

Fluorescence-activated cell sorting (FACS) analysis. Cultured cells were washed twice with cold phosphate-buffered saline (PBS). The cells were then stained with TP80 (a mouse monoclonal anti-human platelet GPIIb-IIIa complex antibody conjugated with fluorescein isothiocyanate; Nichirei, Tokyo, Japan). The cells then were stained with TP80 (a mouse monoclonal anti-human platelet GPIIb-IIIa complex antibody conjugated with fluorescein isothiocyanate; Nichirei, Tokyo, Japan). The cells then were stained with TP80 (a mouse monoclonal anti-human platelet GPIIb-IIIa complex antibody conjugated with fluorescein isothiocyanate; Nichirei, Tokyo, Japan). The cells then were stained with TP80 (a mouse monoclonal anti-human platelet GPIIb-IIIa complex antibody conjugated with fluorescein isothiocyanate; Nichirei, Tokyo, Japan). The cells then were stained with TP80 (a mouse monoclonal anti-human platelet GPIIb-IIIa complex antibody conjugated with fluorescein isothiocyanate; Nichirei, Tokyo, Japan).

Assay for hemoglobin (Hb). The Hb content of HEL cells was measured by a colorimetric method using 2,7-diaminoflcorine.31

RESULTS

Expression of the HOX4A gene in human hematopoietic cell lines. Expression of the HOX4A gene in the promyelocytic leukemia HL60 and erythroleukemia HEL and K562 cells was examined by RT-PCR. Because the primer pair was designed to span an intron on the HOX4A gene, the RT-PCR product of the HOX4A mRNA was predicted to be 434 bp in size (Fig 1). The cytoplasmic RNA was reverse-transcribed, and the cDNA obtained was amplified by 50
cycles of PCR. The RT-PCR products were separated by 2% agarose gel electrophoresis, and the predicted 434-bp fragments were obtained from both the erythroleukemia HEL and K562 cells but not from the promyelocytic leukemia HL-60 cells (Fig 2A). The specificity of the RT-PCR products was confirmed by Southern blotting (Fig 2B), using 32P-labeled 529-bp HOX4A genomic DNA as a probe (probe A shown in Fig 1). In contrast, the 376-bp RT-PCR products of β-actin mRNA were observed in all cell lines examined (Fig 2C). The 434-bp fragments were cloned directly into the pT7Blue T-vector, and the nucleotide sequence was determined. The sequence matched exactly to the predicted HOX4A cDNA sequence.

Introduction of the pMAMneo-HOX4A gene into HEL cells and expression of the HOX4A gene in the transfectants. To investigate the function of the HOX4A gene in cell proliferation and differentiation, we attempted to overexpress the HOX4A gene in HEL cells. The 3.4-kb HOX4A genomic DNA, containing the complete coding region and intron, was inserted into a mammalian expression vector, pMAMneo, which possesses both the Rous sarcoma virus and dexamethasone-inducible mouse mammary tumor virus long terminal repeat promoters. For an experimental transfection, the HOX4A genomic DNA was cloned into pMAMneo in the sense orientation [pMAMneo-HOX4A(+)]. As a control transfection, the fragment was inserted into pMAMneo in the antisense orientation [pMAMneo-HOX4A(-)]. These pMAMneo-HOX4A(+) and pMAMneo-HOX4A(-) plasmid constructs were transfected into HEL cells by means of Lipofectin, after which the HEL cells were cultured in neomycin (G-418)-containing medium for 2 weeks. In both cases, about 20 G418-resistant colonies were obtained when 10 μg of the plasmid DNA were transfected. When only 5 μg of DNA were transfected, colony development did not occur. From these colonies, 10 independent sense or antisense transfectants were isolated.

Cytoplasmic RNA was obtained from the parental HEL cells, the pMAMneo-HOX4A(+)–transfected HEL cells (sense transfectants), and the pMAMneo-HOX4A(-)–transfected HEL cells (antisense transfectants). The RNA was reverse-transcribed, and the obtained cDNA was amplified by 35 cycles of PCR. The RT-PCR products were separated by 7.5% PAGE (Fig 3A). In the sense transfectants, higher levels of HOX4A expression were found in the E1 and E6 cells than in the other transfectants or the parental HEL cells. Generation of a large amount of 434-bp RT-PCR products in the E1 and E6 transfectants indicates that the HOX4A mRNA transcribed from the exogenously introduced HOX4A gene was spliced in the same manner as endogenous HOX4A mRNA. The intensities of the RT-PCR products of the HOX4A mRNA from the C1- and C2-transfected cells and parental HEL cells were compared. The intensity of C2 was approximately equal, and that of C1 appeared to be lower than that of the parental HEL cells. In contrast, the intensity of the 376-bp RT-PCR products of β-actin mRNA did not differ between the parental HEL cells and the transfectants (Fig 3B). To make a quantitative comparison of the mRNA amounts for the HOX4A gene between the parental HEL cells and the E1 and E6 transfectants, we performed PCR at varying numbers of cycles. The PCR products, which were separated on 2% agarose gels, were blotted onto nylon filters and hybridized with a 32P-labeled DNA probe.
REGULATION OF CELL ADHESION BY HOX4A

Fig 3. Induction of exogenous HOX4A gene expression in parental HEL cells and neomycine-selected transfectants. The parental HEL cells (pHEL), two cell clones derived from HEL cells transfected with pMAMneo-HOX4A(+) (E1 and E6), and two cell clones derived from HEL cells transfected with pMAMneo-HOX4A(-) (C1 and C2) were cultured in the presence of dexamethasone (2 μmol/L) for 5 days. Cytoplasmic RNA was isolated from cells of each cell line, subjected to RT-PCR, and analyzed by 7.5% PAGE. Track (M) shows DNA size markers. (A) Detection of HOX4A mRNA is shown. After 35 cycles of amplification with the primers for HOX4A cDNA, samples were electrophoresed and stained with ethidium bromide. (B) Detection of cytoplasmic β-actin mRNA is shown. After 25 cycles of amplification with the primers for β-actin cDNA, samples were electrophoresed and stained with ethidium bromide.

Fig 4. Estimation of the relative amounts of HOX4A mRNA in the parental HEL (pHEL) cells and the E1 and E6 transfectants. (A) Southern blot hybridization is shown. One microgram of cytoplasmic RNA prepared from each group of cells was subjected to RT-PCR with the primers for HOX4A cDNA at 20, 25, 30, and 35 cycles. The amplified DNA fragments were separated on 2% agarose gels, blotted onto nylon filters, and then hybridized with a 32P-labeled probe A. (B) Kinetics of PCR reactions at different cycles are shown. The radioactivity for each signal on the filters was measured by a Bio-Image Analyzer and plotted against the cycle numbers. Symbols (●), (○), and (△) represent the radioactivity of signals derived from the pHEL, E1, and E6 cells, respectively. The dotted line represents 10-fold the HOX4A mRNA of the parental HEL cells.
tants to adhere to untreated, fibronectin-coated, and collagen-coated tissue-culture dishes (Fig 6). Cell attachment, at 37°C, was allowed to occur for 15 minutes, 90 minutes, and 16 hours. The 16-hour experiment clearly showed that the E1 and E6 cells adhered more efficiently to the tissue-culture dishes than the C1, C2, and parental HEL cells (Fig 6A). When the cells were allowed to attach for 90 minutes, the number of E1 and E6 cells adherent to a collagen matrix was markedly greater than the C1, C2, and parental HEL cells (Fig 6B). It was found that the avidity of adhesion of the E1 and E6 cells for fibronectin was stronger than that of the C1, C2, and parental HEL cells, even after just 15 minutes of attachment (Fig 6C). Almost all the E1 and E6 cells and 70% to 80% of the C1, C2, and parental HEL cells attached to fibronectin after 90 minutes and 16 hours. Thus, the adhesive reactions of these cells on a fibronectin matrix reached the saturation point within 90 minutes. Furthermore, the adhesive properties between the transfectants and randomly isolated 12 native HEL cell clones were compared. As shown in Table 1, the E1 and E6 transfectants overexpressing the HOX4A gene were more adhesive than all the native HEL cell clones examined. The above results indicate that cell-extracellular matrix adhesion was increased in the sense transfectants overexpressing the HOX4A gene.

As for the rate of cell growth, there were no significant differences between the transfectants. The Hb contents (μg/10⁶ cells) of the parental HEL, E1, E6, C1, and C2 cells were 0.60, 0.49, 0.53, 0.52, and 0.60, respectively, indicating that high expression of the HOX4A gene had little effect on Hb synthesis.

Expression of integrin molecules in the parental and HOX4A-transfected HEL cells. To examine the possible involvement of integrin molecules in the promotion of cell adhesion, the levels of the integrin αIIb, β1, and β3 subunit mRNA in the parental and HOX4A-transfected HEL cells were compared by RT-PCR and RNA hybridization analyses. The RT-PCR data suggested increased levels of integrin β3 mRNA in E1 and E6, whereas integrin αIIb and β1 mRNA were comparable in the parental HEL, E1, E6, C1, and C2 cells (data not shown). The results of the RT-PCR tests were confirmed by Northern blot analysis, using cDNA fragments of integrin αIIb, β1, and β3 as probes. A distinct correlation between the levels of HOX4A and integrin αIIb and β1 gene expression was not found (Fig 7A and B). In contrast, 6.0-kb hybridizing signals corresponding to integrin β3 mRNA were clearly observed in E1 and E6, whereas the signals were very weak or undetectable in the C1, C2, and parental HEL cells (Fig 7C). In addition, we performed FACS analysis using a monoclonal antibody against human platelet integrin αIIb/β3 (GPIIb-IIIa) complex. As shown in
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leukemia HL-60 cells. This expression pattern is similar to that of HOX-2 homeobox genes because the HOX-2 genes are actively expressed in HEL and K562 cells but not in HL-60 cells. However, compared with the gene expression of HOX-2 loci in HEL and K562 cells, the level of HOX4A expression appeared to be very low in these cells, because we could not detect their transcripts by Northern blot analysis.

To clarify the function of the HOX4A gene, we obtained stable HOX4A transfectants by introducing HOX4A sense or antisense constructs into HEL and K562 cells. Distinct morphologic changes were induced by overexpression of the HOX4A gene in the HEL transfectants, but little occurred in the K562 transfectants. The sense HEL transfectants with high levels of HOX4A mRNA showed aggregate formation. The aggregates of the sense transfectants were composed of densely associated cells and adhered to tissue-culture dishes, whereas the parental HEL cells and antisense transfectants attached poorly to tissue-culture dishes. The property of aggregate formation was shown by cells maintained in continuous culture for over a year, suggesting that the exogenous

Fig 6. Adhesion of parental and transfectant cells to tissue-culture (surface negatively charged), fibronectin-coated, and collagen-coated dishes. Cells, treated with 2 μmol/L of dexamethasone for 3 days were collected, seeded (4 x 10⁵ cells/mL) in tissue-culture, fibronectin-coated, and collagen-coated dishes (3.5 cm in diameter), and cultured in RPMI-1640 containing 10% fetal bovine serum in the presence of 2 μmol/L of dexamethasone. Cell attachment at 37°C for 16 hours (A), 90 minutes (B), and 15 minutes (C) is shown. Nonadherent cells were collected by washing culture flasks twice with culture medium; adherent cells were removed by washing with PBS solution containing 0.2% EDTA. The number of cells in each population was counted, and the percentage of adherent cells per total cells was calculated. Cross bars in (A) and (C) represent standard deviations (SD) of two independent experiments.

DISCUSSION

Many homeobox genes of the human HOX-1, HOX-2, and HOX-3 loci are expressed in hematopoietic cell lines in lineage-restricted patterns. In contrast, almost the entire HOX-4 locus has been silent in the hematopoietic cell lines analyzed to date.

In the present study, we showed by RT-PCR that the HOX4A gene was expressed in the erythroleukemia HEL and K562 cells but not in the promyelocytic

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<th>Clone</th>
<th>Expression</th>
<th>Level of % Adhesion</th>
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<th>% Adhesion to Tissue-Culture Flasks</th>
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* HOX4A gene expression confirmed by RT-PCR. The low levels in native HEL cell clones were similar to that of the parental HEL cells shown in Fig. 3.
† Cells (4 x 10⁵/mL) were seeded in fibronectin-coated dishes (3.5 cm in diameter). Cell attachment at 37°C for 15 minutes. Nonadherent and adherent cells were collected, and percentage of adherent cells per total cells was calculated. Each value represents the mean ± SD of two independent experiments.
‡ Cells (1.8 x 10⁵/mL) were seeded in tissue-culture flasks (25 cm²). Cell attachment at 37°C for 24 hours, and percentage of adherent cells per total cells was calculated. Each value represents the mean ± SD of two independent experiments.
§ Cells (5 x 10⁵/mL) were seeded in tissue-culture flasks (25 cm²). Aggregate formation was scored after 24 hours of incubation at 37°C. ¶ For E1 and E6 clones, the values shown for percentage of adhesion to fibronectin-coated dishes and to tissue-culture flasks represent the mean ± SD of 5 and 3 independent experiments, respectively.
**HOX4A** gene was stably integrated into the chromosomal DNA in the sense transfectants. Furthermore, we found that the sense transfectants overexpressing the **HOX4A** gene adhered more efficiently to fibronectin, collagen, and tissue-culture dishes than did the antisense transfectants and parental HEL cells. In addition, the sense transfectants with high levels of **HOX4A** mRNA were more adhesive than native HEL cell clones with low levels of **HOX4A** mRNA. These findings indicate that overexpression of the **HOX4A** gene enhanced cell-extracellular matrix adhesion by mediating interactions between receptors and ligands. In contrast, Shen et al. reported that overexpression of the **HOX2B** gene in HEL and K562 cells was associated with loss of erythroid features in both cell lines. Because they did not find an alteration of cell adhesion properties in HEL and K562 cells overexpressing either the **HOX2A** or **HOX2B** gene, regulation of cell adhesiveness might be a specific function of the **HOX4A** gene.

The integrins, a family of cell surface adhesion receptors, are composed of noncovalently associated α and β subunits and play a prominent role in mediating cell-cell and cell-extracellular matrix attachments. The HEL cell line expresses several integrin complexes: the α5β1 integrin (a fibronectin receptor), the αVβ3 integrin (a vitronectin receptor), and the αIIbβ3 integrin (the platelet GPIIb-IIIa which acts as a fibrinogen and fibronectin receptor). Northern blot analysis showed that mRNA levels of integrin β3 were significantly increased in the sense transfectants overexpressing the **HOX4A** gene, whereas the levels of integrin αIIb and β1 mRNA did not have a distinct relation to those of the **HOX4A** mRNA. Because we could not detect integrin α5 and αV mRNA in the transfectants and parental HEL cells by Northern blot hybridization, the levels of α5 and αV mRNA were clearly lower than those of αIIb and β1 mRNA in these cells. In addition, FACS analysis showed that the HEL cells with high levels of **HOX4A** mRNA expressed increased levels of GPIIb-IIIa complex. This elevation of cell surface expression of GPIIb-IIIa is consistent with the enhancement of cell-fibronectin matrix adhesion in the sense transfectants overexpressing the **HOX4A** gene. In contrast to the expression of GPIIb-IIIa in HEL cells, K562 cells have been reported to lack integrin αIIb expression. This may be why overexpression of the **HOX4A** gene in the K562 transfectants did not induce morphologic changes. These findings indicate that overexpression of the **HOX4A** gene leads to elevation of integrin β3 subunit mRNA and suggest that the **HOX4A** protein either induces regulatory proteins for the activation of the integrin β3 gene or directly transactivates the β3 gene.

There is increasing evidence that the expression of homeobox genes is involved in cell adhesion of hematopoietic cells. Enforced expression of the murine Hlx, a diverged homeobox gene, in myeloid FDC-P1 cells promoted changes in colony morphology from a compact to a diffused type. Ectopic Hlx expression in the T-cell line Tikaut induced changes in both colony morphology and cell adhesiveness, concomitant with decreased expression of adhesion molecules ICAM-1 (CD54) and Pgp-1 (CD44). Abnormal expression of the Hox-2.4 gene in mouse myeloid leukemia cells blocked their differentiation into macrophages after stimulation by interleukin-6. This disruption was characterized by a reduction in the number of adherent cells. The colony formation of erythroid progenitor cells, at the stages of burst forming unit-erythroid and colony forming unit-erythroid, is enhanced by activin A, a member of the transforming growth factor-β family of cell surface adhesion receptors, and the platelet GPIIb-IIIa complex.
forming growth factor-β superfamily, which is a potent activator of homeobox genes in a mesoderm-inducing system. Based on these previous studies and our present findings, it is very likely that homeodomain proteins, including the HOX4A protein, are involved in the gene expression of cell adhesion molecules in hematopoietic progenitor cells.

A hematopoietic inductive microenvironment (bone marrow stromal cells) is necessary for hematopoiesis in vivo and to support the proliferation and differentiation of hematopoietic progenitor cells in vitro. Interactions between stromal and hematopoietic cells through the agency of an extracellular matrix are thought to be important for the regulation of hematopoietic stem cell proliferation and differentiation. Patel and Lodish have observed that murine erythrocyte (MEL) cells, induced by dimethyl sulfoxide on fibronectin-coated dishes, differentiate as far as enucleating erythrocytes, suggesting that a fibronectin matrix is a permissive microenvironment where erythroid precursor cells can proliferate, migrate, and express their normal differentiation program. Our results have provided definitive evidence that overexpression of the HOX4A gene in HEL cells enhances cell-fibronectin matrix adhesion and, coincidentally, elevates expression of the integrin β3 gene, which encodes a component of the receptor for fibronectin. So far, there has been little information about the function of the HOX4A gene. Based on the present work, we propose that expression of the HOX4A gene is closely associated with interactions between cells and extracellular matrices.

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REFERENCES

36. Ylanne J, Cheresh DA, Virtanen I: Localization of $\beta_1$, $\beta_5$, $\alpha_v$, $\alpha_v$, and $\alpha_v$ subunits of the integrin family in spreading human erythroleukemia cells. Blood 76:570, 1990
43. Rosa FM: Mix. 1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of Xenopus embryos. Cell 57:965, 1989
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